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TANGERETIN INHIBITS THE PROLIFERATION OF
HUMAN BREAST CANCER CELLS VIA CYP1A1/CYP1B1
ENZYME INDUCTION AND CYP1A1/CYP1B1–MEDIATED
METABOLISM TO THE PRODUCT 4' HYDROXY
TANGERETIN

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Abstract

Tangeretin is a polymethoxylated flavone with multifaceted anticancer activity. In the present study, the metabolism and further antiproliferative activity of tangeretin was evaluated in the CYP1 expressing human breast cancer cell lines MCF7 and MDA-MB-468 and the normal breast cell line MCF10A. Tangeretin was converted to 4' OH tangeretin by recombinant CYP1 enzymes and in MCF7 and MDA-MB-468 cells. This metabolite was absent in MCF10A cells that did not express CYP1 enzymes. Tangeretin exhibited submicromolar IC₅₀ ($0.25 \pm 0.15 \mu\text{M}$) in MDA-MB-468 cells, whereas it was less active in MCF7 cells ($13.5 \pm 0.8 \mu\text{M}$) and completely inactive in MCF10A cells ($>100 \mu\text{M}$). In MDA-MB-468 cells that were coincubated with the CYP1 inhibitor acacetin, an approximately 70-fold increase was noted in the IC₅₀ ($18 \pm 1.6 \mu\text{M}$) of tangeretin. In the presence of the CYP1 inhibitor acacetin, the conversion of tangeretin to 4' OH tangeretin was significantly reduced in MDA-MB-468 cells ($2.55 \pm 0.19 \mu\text{M}$ vs. $6.33 \pm 0.12 \mu\text{M}$). The mechanism of antiproliferative action involved cell cycle arrest at the G1 phase for MCF7 and MDA-MB-468 cells, whereas the cell cycle of MCF10A cells was unaffected by $10 \mu\text{M}$ of tangeretin treatment for 24 and/or 48 h. Tangeretin was further shown to induce CYP1 enzyme activity and CYP1A1/CYP1B1 protein expression in MCF7 and MDA-MB-468 cells. Taken collectively, the results suggest that tangeretin inhibits the proliferation of human breast cancer cells via CYP1A1/CYP1B1 enzyme induction and CYP1A1/CYP1B1-mediated metabolism to the product 4' hydroxy tangeretin.

Abbreviations

CYP1, cytochrome P450 CYP1B1, CYP1A1 and CYP1A2; HPLC, high pressure liquid chromatography; IC₅₀, 50% inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;

Keywords

Flavonoids, tangeretin, cytochrome P450 CYP1 enzymes, antiproliferation, breast cancer

1. Introduction

Flavonoids are polyphenolic molecules with multiple modes of biological action. Their cancer preventative and/or anticancer activity has been attributed to various different mechanisms including the induction of apoptosis, the modulation of cell signaling, the inhibition of the cell cycle and notably the process of mitosis, the induction and/or modulation of autophagy, the inhibition of invasion and metastasis and the interaction with Reactive Oxygen Species (ROS) and ROS signaling (Johnson and de Mejia, 2013; Qi et al., 2015; Hsieh et al., 2016; Cheng et al., 2015; Salmela et al., 2012; Sosa et al., 2013; Kim et al., 2015; Jo et al., 2015; Li et al., 2013; Doleckova et al., 2012). In addition to the aforementioned biological activities, the interactions of natural dietary flavonoids with xenobiotic phase I metabolizing enzymes, notably the cytochrome P450 CYP1 enzymes, have been investigated by recent studies (Androutsopoulos et al., 2008; Androutsopoulos et al., 2009a; Androutsopoulos et al., 2009b; Androutsopoulos et al., 2009c; Androutsopoulos et al., 2009d; Androutsopoulos et al., 2010; Androutsopoulos and Spandidos 2013; Androutsopoulos and Tsatsakis 2014). CYP1 enzymes have been shown to participate in the metabolic activation of pro-carcinogens to their ultimate carcinogenic derivatives, whereas recently an anti-inflammatory role of these enzymes was demonstrated in Cyp1a1/1a2/1b1 ($-/-$) C57BL/6J triple knockout mice compared with C57BL/6J wild-type mice, via the regulation of the metabolism of arachidonic acid lipid mediators of inflammation (Nebert and Dalton 2006; Divanovic et al., 2013). With regard to dietary flavonoids, a cancer therapeutic effect of these compounds has been demonstrated in breast and liver cancer cells via their intracellular metabolism by CYP1 enzymes (Androutsopoulos et al., 2008; Androutsopoulos et al., 2009a; Androutsopoulos et al., 2009b; Androutsopoulos et al., 2009d). These compounds are

oxidized to their corresponding hydroxylated derivatives via aromatic hydroxylation and/or demethylation reactions occurring at the B and/or A rings of the polyphenolic moiety (Androutsopoulos et al., 2008; Androutsopoulos et al., 2009a; Androutsopoulos et al., 2009b; Androutsopoulos et al., 2009d; Androutsopoulos and Spandidos 2013; Androutsopoulos and Tsatsakis 2014). The resulting metabolites inhibit cancer cell growth at equivalent and/or higher levels compared with the corresponding parent compounds (Androutsopoulos et al., 2008; Androutsopoulos et al., 2009a; Androutsopoulos et al., 2009b; Androutsopoulos et al., 2009d). It is important to note that synergistic effects resulting from the combined action of the parent flavonoid compound and the CYP1-metabolite have been documented, thus accounting for the overall antiproliferative activity (Androutsopoulos and Spandidos 2013).

Tangeretin is a fully methoxylated flavone that is present in the peel of citrus fruits and has demonstrated anticancer activity via biologically distinct mechanisms of action. Tangeretin causes G2/M arrest and induces apoptosis in glioma cells by modulating PTEN and the cell cycle regulating genes, namely D1 and cdc2, whereas in human meningioma cells this compound induces apoptosis by enhanced phosphorylation of glycogen synthase 3 β (GSK3 β) and inhibition of the Wnt5/ β -catenin pathway (Ma et al., 2016; Das et al., 2015). Tangeretin was further shown to inhibit breast cancer formation in a rat model of DMBA-induced mammary carcinogenesis via p53/p21 upregulation and inhibition of metastasis by downregulation of MMP2, MMP9 and VEGF expression (Arivazhagan and Sorimuthu Pillai 2014; Lakshmi and Subramanian 2014). Despite these promising studies, the metabolism of tangeretin by CYP1 enzymes remains poorly defined. With the exception of a previous study by Walle and Walle that examined tangeretin

metabolism by recombinant CYP enzymes and the hepatic liver S9 fraction, there is no evidence in the literature regarding the association of CYP1 enzymes and the cancer therapeutic effect of tangeretin (Walle and Walle, 2007). In the present study, the metabolism of tangeretin was investigated in the CYP1 expressing breast cancer cell lines MCF7 and MDA–MB–468 and the normal breast cell line MCF10A. The data suggest that tangeretin is metabolized to one major conversion product by a demethylation reaction at the B ring that in turn inhibits cancer cell growth.

2. Materials and Methods

2.1 Chemicals and antibodies

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), 7-ethoxyresorufin, resorufin, α -naphthoflavone, acacetin, tissue culture reagents and media, Western blotting lysis buffer and DTT were purchased from Sigma Aldrich (St Louis, MO, USA). Tangeretin and 4' OH tangeretin were purchased from Apin chemicals (Abingdon, UK). Western blotting reagents were from Bio-Rad (Berkeley, CA, USA). The polyclonal antibody for CYP1A1 was from Daiichi Pure Chemicals (Gentest corporation, MA, USA), whereas the monoclonal antibodies for CYP1B1 from Auvation Limited (Glasgow, Scotland, UK), and for β -actin from Cell signaling (Leiden, Netherlands). Secondary antibodies for western blotting were from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2 Cell culture

MCF7 and MDA–MB–468 cells were maintained in RPMI with glutamine (2 mM) containing 10% heat-inactivated FBS and penicillin/streptomycin. MDA–MB–468

cells were grown in RPMI without phenol red, whereas MCF10A cells were grown in DMEM:F12 with insulin (10 μ g/ml), hydrocortisone (500 ng/ml), EGF (20 ng/ml), 10% FBS and 2 mM glutamine. The cells were grown in a humidified incubator at 37 °C in 5% CO₂ /95% air and passaged every 3 to 4 days using trypsin EDTA (0.25% v/v).

2.3 Tangeretin metabolism

Recombinant CYP1 enzymes and control microsomes were incubated with 10 μ M of tangeretin at 37 °C in the presence of NADPH (0.5 mM), MgCl₂ (0.5 mM) and phosphate buffer (20 mM). Time points were obtained at 0, 5, 10, 15, 20 and 25 min intervals. The reactions were terminated by addition of equal volumes of methanol and acetic acid at a 100:1 ratio. The samples were centrifuged at 3,500 g for 20 min at 4 °C and the supernatants were analyzed by reversed phase HPLC.

2.4 HPLC analysis

The methodology has been described in detail in previous publications (Androutsopoulos et al., 2009a, Androutsopoulos et al., 2008). A Luna C18 4.6 x 150 mm 5 μ column was used and the mobile phase consisted of solvents A and B. Solvent A comprised 1% acetonitrile and 0.5% acetic acid in H₂O and solvent B 4% acetonitrile and 0.5% acetic acid in CH₃OH. The following gradient was used: 60% solvent A and 40% solvent B at time=0 min and 10% solvent A and 90% solvent B at time=10 min. The final conditions were maintained for 1 min and the composition of the solvents was adjusted to the initial conditions with 8 min remaining for column equilibration after each run. The detection of tangeretin concentration was monitored using a Waters Series 200 UV detector (Waters, Hertfordshire, UK) at 327 nm. The concentration of tangeretin was estimated by a calibration curve covering the range of

0.05–10 μ M. The assay was carried out at 37 °C and the flow rate was 1 ml/min. The average recovery for tangeretin was 93% and the retention time using the aforementioned parameters was 18.1 min.

2.5 LC-MS analysis

Mass spectrometry analysis of tangeretin was conducted on an Agilent 1100 Series LC/MSD Trap XCT systems with a photodiode array detector. The separation of tangeretin was conducted using a Phenomenex Luna 5 μ C18 (250 \times 4.6mm) column and the following solvents: (A) water, (B) methanol and (C) acetonitrile. The initial conditions were as follows: 59% solvent A, 40% solvent B and 1% solvent C. The analytical column temperature was 40°C and the flow rate was 0.8 ml/min. A total of 50 μ L of sample solution were analyzed. A linear gradient was initiated immediately on the start of the analysis with solvent B rising to 90% and solvent C rising to 4% over 15 min. These solvent compositions were held for 2min before returning to the initial conditions (using a flow rate of 0.8ml/min). A re-equilibration time of 8 min was allowed between each sample analysis. The photodiode array detector was set at 330 nm. The LC flow was controlled from the photodiode array detector into the mass spectrometer without stream splitting. The MS determination was acquired in electrospray ionisation (ESI) positive mode. Molecular ions ($[M+H]^+$) were detected in the range of 200–500 using an auto-MS mode. Nitrogen was used as the nebulizing gas at 60 psi and as drying gas with the flow rate of 10 L/min at 350°C. The MS data were analyzed using LC/MSD Trap software 5.3. The Retention time (R_t) of tangeretin under these conditions was 17.9 min.

2.6 MTT assay

MCF7, MDA-MB-468 and MCF10A cells (1×10^4 cells/ml) were seeded in 96-well plates and the antiproliferative effect of tangeretin was examined as described previously (Androutsopoulos et al., 2008). Inhibition experiments were conducted in the presence of 0.5-1 μ M α -naphthoflavone and/or acacetin.

2.7 FACS analysis

MDA-MB-468 and MCF10A were grown in T25 flasks at approximately 30% confluence. The cells were treated with tangeretin at concentrations of 10, 30 and 50 μ M in the presence and/or absence of CYP1 inhibitors (0.5–1 μ M) for 24 and 48 h. The cells were subsequently washed with PBS, removed by trypsin/EDTA and centrifuged at 3,500 rpm for 5 min. The cells were fixed in 70% ethanol for at least 24 h at -20 °C. The ethanol was removed and PBS containing PI (50 μ g/ml) and RNase (100 μ g/ml) was added to the cells that were incubated further for 30 min at 37 °C. Flow cytometry was conducted using a Beckman Coulter flow cytometer at PMT4 and at least 10,000 events were acquired for analysis. The Multicycle analysis 2.0 Software was used for the determination of the cell cycle corresponding to the samples.

2.8 Western blotting

MCF7, MDA-MB-468 and MCF10A cells were cultured in T25 flasks at a density of 5×10^5 cells/ml. The cells were washed once with PBS and lysed with 100 μ l of lysis buffer that contained protease inhibitor cocktail and DL-dithiothreitol (DTT, 1 mM). The cells were subsequently sonicated on ice for 5 min and centrifuged at 13,000 rpm at 4 °C for 15 min. The protein concentration required for the experiment was

adjusted to 0.7 mg/ml for each sample, and the protein extract was mixed with sample buffer that contained 5% mercaptoethanol at a 1:1 ratio. The samples were loaded on an acrylamide gel containing 10% acrylamide for the resolving gel and 5% acrylamide for the stacking gel and electrophoresis was carried out for 1 h at 120 V. Following electrophoresis, the proteins were transferred by wet blotting to a PVDF membrane. The membrane was incubated in 10% milk/0.05% TBST at room temperature for 1 h by continuous shaking in order to block the non specific binding sites. The primary antibodies against CYP1A1, CYP1B1 and β -actin were added to the membrane at 1:800, 1:500 and 1: 3,000 dilutions, respectively at 4 °C overnight. The membrane was subsequently washed three times with 0.05% TBST and incubated with the secondary antibody against HRP (1:2,000) diluted in 1% milk/0.05% TBST at room temperature for 1.5 h. The membrane was finally exposed to ECL reagents, and the expression profile of the proteins was developed on film.

2.9 EROD assay

MCF7 and/or MDA-MB-468 cells (2×10^5 cells/ml) were plated in 96-well plates. The cells were incubated at 37°C, 5% CO₂ for 24h and subsequently tangeretin was added at a concentration range of 0.1, 0.3, 1, 3, 10, 30 and 100 μ M, respectively. The induction of EROD activity was assayed at the 6, 9 and 24 h time points and at shorter time points, namely 15,30 and 60 min. Following the end of the treatment period, the medium was aspirated and the cells were washed with PBS thrice. A total of 200 μ l of medium containing 5 μ M 7-ethoxyresorufin and 1.5mM salicylamide to inhibit conjugating enzymes were added to each well, and the plates were further incubated for 1 h. The reactions were terminated by addition of 150 μ l of ice-cold methanol to the wells, followed by centrifugation at 3,500 rpm at 4°C for 15min. Subsequently, 200 μ l of supernatant were transferred to a clear bottom black 96-well plate and the amount of

resorufin produced was measured using a fluorescence plate reader with an excitation and emission wavelength at 530nm and 590nm, respectively. The data were analyzed, following subtraction of the blank fluorescence that contained resorufin alone from each sample reading. The concentration of resorufin was measured using a calibration curve. The units of enzyme activity were expressed as concentration of resorufin formed per time, per amount of cells.

2.10 Statistical analysis

The data were presented as the mean of at least three independent measurements and were analyzed by the paired t-test, the unpaired t-test and the One-Way analysis of variance using GraphPadPrism. Error bars represent mean \pm STDEV for at least n=3 determinations.

3. Results

3.1 Recombinant CYP1 enzymes metabolize tangeretin

The metabolism of tangeretin by recombinant CYP1 enzymes was examined by LC-MS analysis. A main metabolite was formed, which was assigned as TP1. The mass spectrum of TP1 indicated the presence of an ion at 359.1, whereas the ion that corresponded to the parent compound tangeretin was 373.1 (Fig. 1B). CYP1A1 was shown to be the predominant catalyst of tangeretin metabolism compared with CYP1B1 and CYP1A2 following 30 min of enzyme incubation with the compound (Fig. 1A). CYP1B1 was a weak metabolizer of tangeretin and produced a minor quantity of TP1 (Fig. 1A). The order of the overall metabolism was as follows: CYP1A1>CYP1A2>CYP1B1 (Fig. 1A). The difference in the molecular weights of tangeretin and TP1 (373–359=14), as determined by their molecular ions suggested that a de-methoxylation occurred in the initial structure of tangeretin (Fig. 1C). The

comparison with an authentic standard of 4' OH tangeretin revealed that this compound had the same retention time as TP1, thus confirming the identity of this metabolite as 4' OH tangeretin (Fig. 1C).

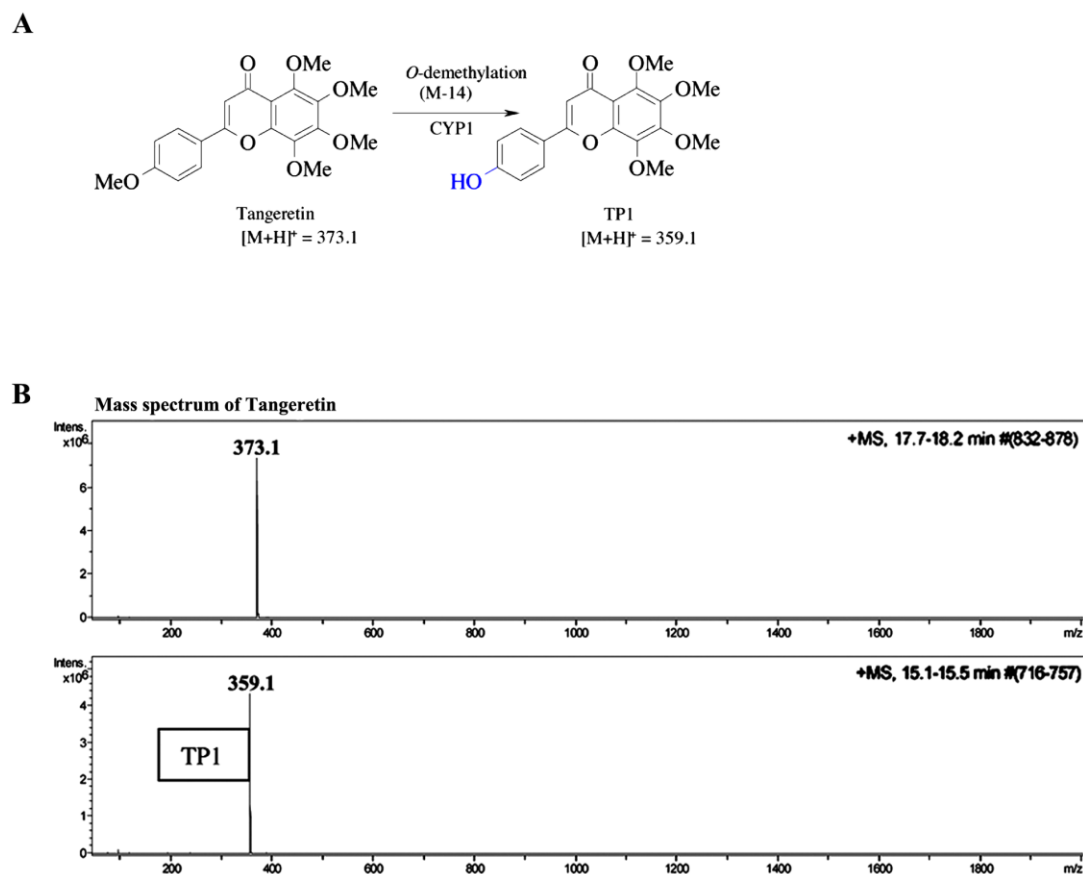


Figure 1. Cytochrome P450 CYP1-mediated metabolism of tangeretin. Recombinant CYP1 enzymes were incubated with tangeretin (10 μ M) and samples were collected at 0, 5, 10, 15, 20, 25 and 30 min time intervals as described in Material and Methods. Control incubations contained control microsomes with empty vector. (A) Schematic bioconversion of tangeretin to the metabolite 4' OH tangeretin and/or TP1. (B) The concentration of tangeretin and its metabolite TP1 was measured by LC-MS analysis and a representative mass spectrum is shown for the 30 min time point.

3.2 CYP1 enzymes metabolize tangeretin in MCF7 and MDA–MB–468 human breast cancer cells

The human breast cancer cell lines MCF7 and MDA–MB–468 have been employed in previous studies for the investigation of the CYP1-mediated metabolism and bioactivation of dietary flavonoids (Androutsopoulos et al., 2008). In the present study, the metabolism of tangeretin was examined in the aforementioned cell lines. A main metabolite that matched the retention time of TP1 was identified following incubation of tangeretin with MDA–MB–468 and/or MCF7 cells for 24 h (Fig. 2A). This metabolite was absent in MCF10A cell extracts (Fig. 2A,B). The reduction of the parent compound was higher in MDA–MB–468 compared with MCF7 cells following 24 h of incubation (Fig. 2A, B). The metabolism of tangeretin to TP1 was enhanced in MCF7 cells that were pretreated with TCDD (10 nM) for 24 h, whereas the metabolism of this compound in MDA–MB–468 cells was evident from 1 h of incubation and gradually increased at the 3, 6 and 9 h time points, respectively (Fig. 2C,D). LC–MS was further used in order to add insight in the identification of the primary metabolite of tangeretin. The LC chromatogram of the assay matched that noted from the HPLC experiments with regard to the retention time of the compounds, whereas the MS analysis revealed that TP1 exhibited a molecular mass of 359 (Fig. 3A,B). TP1 was identified as 4' OH tangeretin by comparison with an authentic standard. Thus, the main metabolite of tangeretin in the CYP1 expressing cell lines MDA–MB–468 and MCF7 was 4' OH tangeretin.

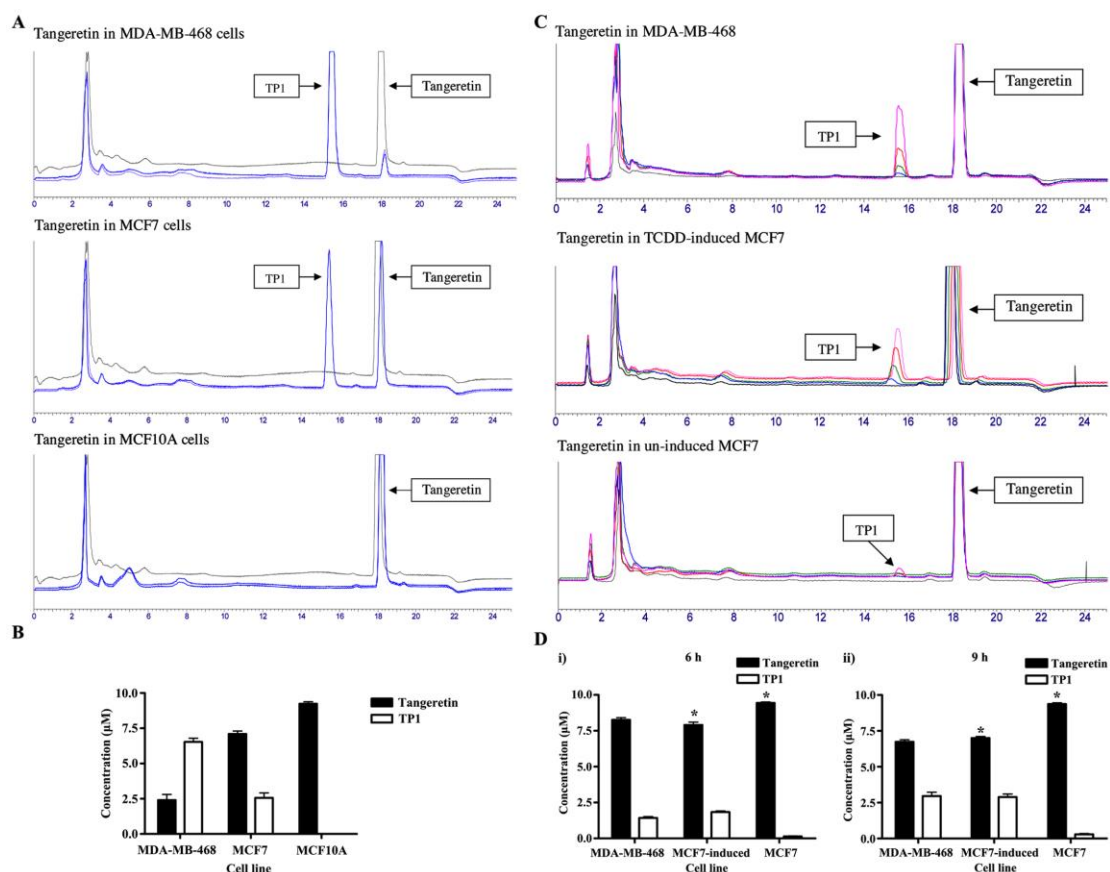


Figure 2. HPLC analysis of tangeretin following incubation with the human breast cell lines MCF7, MDA–MB–468 and MCF10A. A starting concentration of 10 µM was used and the metabolism was investigated at 0, 3, 6, 9 and 24 h time points by HPLC as described in materials and methods. The experiments were conducted at least 3 times and error bars indicate STDEV of the mean. (A) HPLC chromatograms of tangeretin in the 3 breast cell lines at 0 (grey color) and 24 h (blue color) time points. (B) Measurement of the concentration of the parent compound and the metabolite TP1 in the 3 cell lines. (C) HPLC chromatograms of tangeretin in MCF7 cells, MCF7 pretreated for 24 h with 10 nM TCDD, and MDA–MB–468 cells at 0 (grey color), 1 (blue color), 3 (green color), 6 (red color) and 9 (pink color) h time points. (D) Quantification of the concentration of tangeretin and the metabolite TP1 at the 6 and 9 h time points.

3.3 Tangeretin is bioactivated in the CYP1–expressing cell lines MDA–MB–468 and MCF7

Previous studies have demonstrated that MDA–MB–468 cell constitutively express CYP1 enzymes compared with MCF10A cells that express little or no CYP1 protein, whereas MCF7 cells express minimal amounts of CYP1 enzyme levels that are

increased following pretreatment with a CYP1 inducer (Androutsopoulos et al., 2008; Androutsopoulos et al., 2009). The present study utilized the aforementioned cell lines to investigate the antiproliferative activity of tangeretin. This compound was considerably active in MDA–MB–468 cells and indicated a submicromolar IC₅₀ ($0.25 \pm 0.15 \mu\text{M}$), whereas it demonstrated minimal antiproliferative activity in MCF10A cells ($>100 \mu\text{M}$) (Fig. 4A). In MCF7 cells the IC₅₀ of tangeretin ($13.5 \pm 0.8 \mu\text{M}$, Fig. 4B) was considerably higher compared with MDA–MB–468 cells, whereas pretreatment of the cells with TCDD (10 nM) for 24 h decreased the overall IC₅₀ value to a minor extent ($9.7 \pm 0.5 \mu\text{M}$, Fig. 4B) compared with the difference noted in the aforementioned 2 cell lines. Despite this minor decrease, the results were significantly different between the two different types of treatments (MCF7 +– TCDD)(Fig. 4B).

The cytotoxicity of tangeretin in MDA–MB–468 cells was investigated in the presence of the CYP1 inhibitors acacetin and α -naphthoflavone (Fig. 4C). The IC₅₀ of tangeretin was considerably increased from $0.25 \pm 0.15 \mu\text{M}$ to $18 \pm 1.6 \mu\text{M}$ in the presence of the CYP1 inhibitor acacetin (Fig. 4C). Similar results were noted for the use of α -naphthoflavone as a CYP1 inhibitor. The cytotoxicity of the metabolite TP1 was evaluated in the 3 breast cell lines and it was found that this compound was more active in MDA–MB–468 compared with MCF7 and MCF10A cells (Fig. 4D). MCF7 cell viability was reduced to a higher extent by 5, 10 and 20 μM of TP1 compared with that noted for MCF10A cells ($P < 0.05$, Fig. 4D).

In addition, metabolic studies revealed that in the presence of the CYP1 inhibitors the conversion of 4' OH tangeretin from tangeretin was substantially reduced both in MDA–MB–468 and MCF7 cells (Fig. 4E, F). Specifically, an approximately 3–fold decrease ($2.55 \pm 0.19 \mu\text{M}$ vs. $6.33 \pm 0.12 \mu\text{M}$) was noted for the conversion of TP1 by

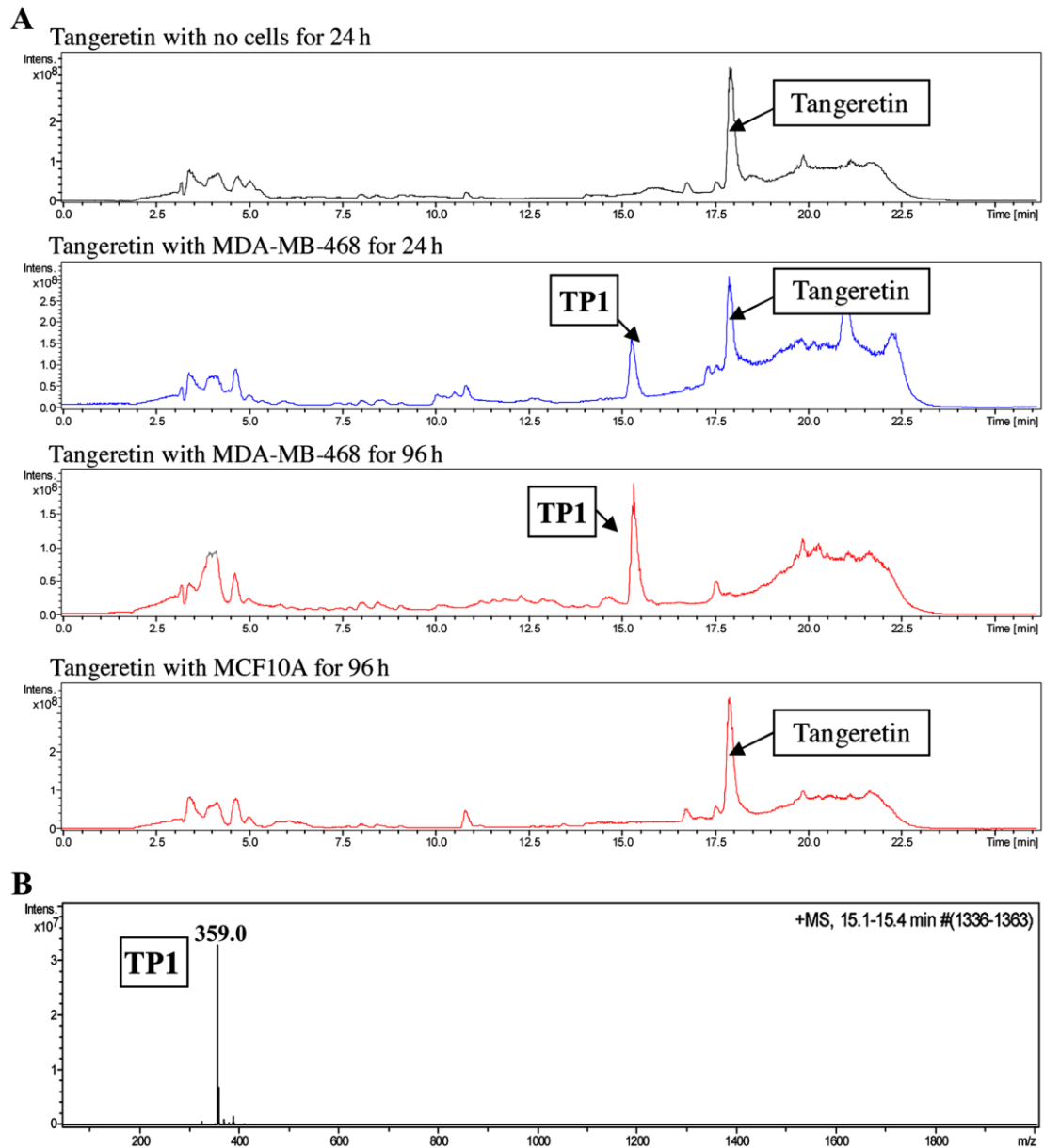


Figure 3. LC-MS analysis of the metabolism of tangeretin in MDA-MB-468 and MCF10A cells. (A) LC chromatograms of the incubation of tangeretin with the MDA-MB-468 and MCF10A cell lines at various time points (24, 96 h). (B) Mass spectrum of TP1 in the cell line incubates.

tangeretin, in the presence of the CYP1 inhibitors compared with the incubation of the parent compound with MDA-MB-468 cells alone (Fig. 4F).

The antiproliferative effect of tangeretin on the 2 human breast cancer cell lines and the normal breast cell line was further investigated by flow cytometry analysis.

Treatment of MDA–MB–468 cells with tangeretin (10 μ M) for 24 and 48 h resulted in a significant increase of the population of cells in the G1 phase (Fig. 5A–D, K, $P < 0.05$). This increase was accompanied by a concomitant significant decrease in the percentage of cells in the S phase in the tangeretin–treated (Fig. 5A–D, K, $P < 0.05$) samples. The effect of tangeretin in MDA–MB–468 cells was partially reversible as in the presence of the CYP1 inhibitor acacetin a significant decrease in the population of the cells at G1 phase was noted compared with the single treatment of tangeretin (Fig. 5A,B,D,K, $P < 0.05$).

MCF7 cells appeared less sensitive to tangeretin treatment and although significant differences were noted between the control and the treated samples, these were somewhat lower in magnitude compared to those noted in MDA–MB–468 cells (Fig. 5 E–G, L). In contrast to the breast cancer cell lines, MCF10A cells were unaffected by tangeretin treatment (10 μ M) at either 24 and/or 48 h (Fig. 5H–J, M).

3.4 Tangeretin induces CYP1 enzyme expression in MCF7 and MDA–MB–468 cells

The initial experiments in MCF7 cells revealed metabolism of tangeretin in the absence of TCDD pretreatment to the conversion product 4' OH tangeretin (Fig. 2A). In addition, the previous study on nobiletin by Surichan and colleagues demonstrated that this structurally similar polymethoxyflavone was capable of inducing its own metabolism in MCF7 cells via induction of CYP1 enzyme expression (Surichan et al., 2012). Based on this evidence, the induction of CYP1 enzyme expression by tangeretin was investigated in MCF7 and MDA–MB–468 cells following treatment with this compound for 24 h. CYP1 enzyme activity was significantly increased in MCF7 and MDA–MB–468 cells following treatment with 3, 10 and 30 μ M of

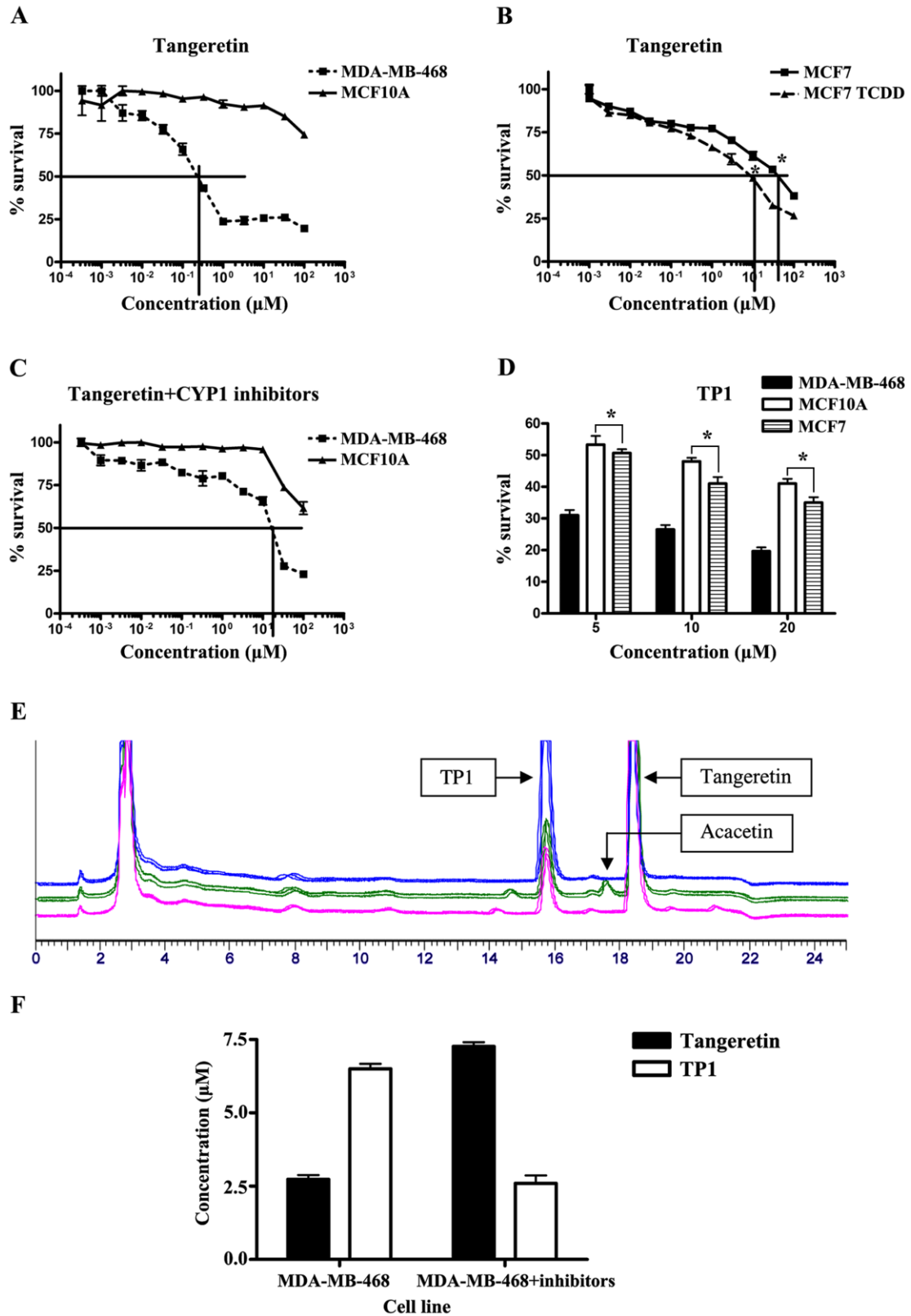


Figure 4. The bioactivation of tangeretin in the CYP1 expressing cell lines MDA–MB–468 and MCF7. Cell viability was measured using the MTT assay as determined in the materials and methods section. The experiments were conducted at least 3 times and error bars indicate STDEV of the mean. Cell survival plots indicating the IC50 determination of tangeretin in (A) MDA–MB–468 and MCF10A cells and (B) MCF7 cells and MCF7 cells pretreated for 24 with 10 nM TCDD, and (C) MDA–MB–468 and MCF10A cells in the presence of the CYP1 inhibitor acacetin (0.5 μ M). Similar results were obtained when the CYP1 inhibitor α -naphthoflavone was used. (D) The inhibition of cell viability in MDA–MB–468, MCF10A and MCF7 cells by the tangeretin–metabolite TP1. TP1 was tested at concentrations of 5, 10 and 20 μ M. * indicate significant differences of the reduction in cell viability between the MCF10A and the MCF7 cells ($P < 0.05$). (E) The metabolism of tangeretin in MDA–MB–468 cells in the presence and/or absence of the CYP1 inhibitor acacetin (0.5 μ M). HPLC chromatogram of MDA–MB–468 cell incubation with tangeretin, in the presence and/or absence of acacetin (0.5 μ M) for 24 h. (F) Quantification of the concentration of the compounds tangeretin and TP1 in MDA–MB–468 cells, in the presence and/or absence of the CYP1 inhibitor acacetin (0.5 μ M).

tangeretin (Fig. 6A,B). In MCF7 cells, CYP1 enzyme activity indicated a substantial increase at 3 μ M that peaked at 10 and 30 μ M of compound treatment compared with control cells (Fig. 6B). In contrast to MCF7, MDA–MB–468 cells exhibited a basal expression of CYP1 enzyme activity that was increased considerably following treatment with 3, 10 and 30 μ M of tangeretin (Fig. 6A). This increase was similar among all treatments, as opposed to MCF7 cells where a dose response was noted for the concentration range 0–10 μ M (Fig. 6B). The maximum activity in MDA–MB–468 cells was nearly half of that noted for 10 nM of TCDD (152 ± 3.7 vs. 225 ± 12.1 pmol/min/ 10^6 cells, Fig. 6A). A similar trend was noted for MCF7 cells, with the exception that the induction caused by TCDD was considerably higher compared with that caused by 30 μ M of tangeretin treatment (53 ± 1.4 vs. 21 ± 1.1 pmol/min/ 10^6 cells, Fig. 6B). At the highest concentration of 30 μ M of tangeretin, an approximately 3–fold and 20–fold increase in CYP1 enzyme activity was observed compared with

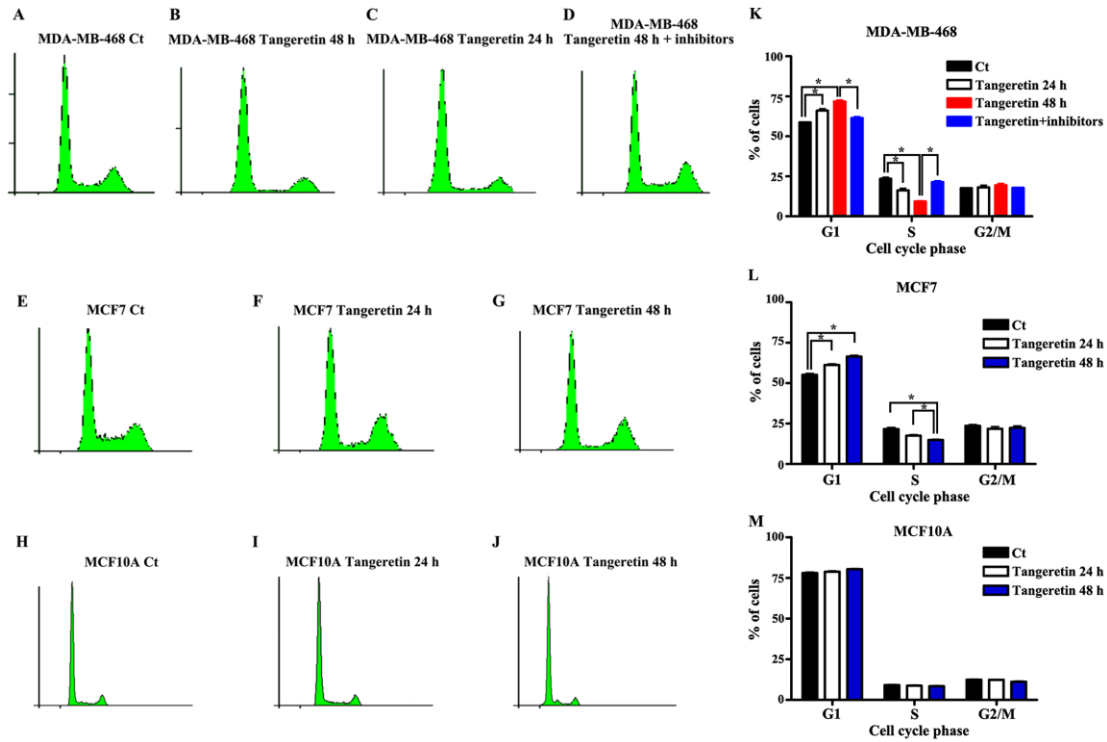


Figure 5. Cell cycle analysis of tangeretin in the 3 human breast cell lines MCF7, MDA-MB-468 and MCF10A at 24 and 48 h time points. Cell cycle histograms of (A) control MDA-MB-468 cells (B) tangeretin-treated (48 h) MDA-MB-468 cells (C) tangeretin-treated (24 h) MDA-MB-468 cells (D) tangeretin-treated (48 h) MDA-MB-468 cells in the presence of the CYP1 inhibitor acacetin (0.5 μ M) (E) control MCF7 cells (F) tangeretin-treated (24 h) MCF7 cells (G) tangeretin-treated (48 h) MCF7 cells (H) control MCF10A cells (I) tangeretin-treated (24 h) MCF10A cells (J) tangeretin-treated (48 h) MCF10A cells. Distribution of the cells in each phase of the cell cycle. (K) MDA-MB-468 cells (L) MCF7 cells (M) MCF10A cells. The experiments were conducted at least 3 times and error bars indicate STDEV of the mean. * indicate significant differences in the cell population of each phase between the control and the treated samples ($P<0.05$). * indicate significant differences in the cell population of each phase between the tangeretin-treated samples in the presence and/or absence of the CYP1 inhibitor acacetin (0.5 μ M) ($P<0.05$).

control cells, for MDA-MB-468 and MCF7 cells, respectively (152 ± 3.7 vs. 55 ± 5.4 pmol/min/ 10^6 cells, 21 ± 1.1 vs. 0.9 ± 0.05 vs. pmol/min/ 10^6 cells Fig. 6A,B).

The induction of CYP1 enzyme expression by tangeretin was further monitored by western immunoblotting. Tangeretin induced CYP1A1 protein in a dose dependent manner in MDA-MB-468 and MCF7 cells (3–30 μ M) (Fig. 6C,D). The induction of

CYP1A1 protein at 3 μ M of tangeretin treatment was lesser in MCF7 compared with MDA-MB-468 cells, whereas the induction of CYP1A1 in MDA-MB-468 cells was approximately half of that noted for TCDD, as opposed to MCF7 cells (Fig. 6C,D). With regard to MCF7 cells, the induction of CYP1A1 by tangeretin was lower than half of that noted for TCDD (Fig. 6D). In contrast to CYP1A1, tangeretin induced CYP1B1 protein expression at minimal levels in MDA-MB-468 cells compared with DMSO control samples (Fig. 6C).

4. Discussion

Tangeretin is a flavonoid that has demonstrated various types of anticancer activities. In the present study, the interactions of cytochrome P450 CYP1 enzymes with tangeretin were investigated. CYP1 enzymes metabolized tangeretin to the conversion product TP1 that was identified as 4'OH tangeretin. CYP1A1 was the main metabolic catalyst followed by CYP1A2 and CYP1B1. In addition, tangeretin was metabolized to 4'OH tangeretin in the human breast cancer cell lines MDA-MB-468 and MCF7, whereas this conversion was absent in the normal breast cell line MCF10A. 4'OH tangeretin exhibited higher antiproliferative activity compared with tangeretin that resulted in the bioactivation of tangeretin in the CYP1 expressing breast cancer cell lines, as demonstrated by metabolic, cell cycle and cytotoxicity studies in the presence of the CYP1 inhibitors acacetin and α -naphthoflavone. Furthermore, tangeretin was capable of inducing CYP1 enzyme activity and CYP1A1/CYP1B1 enzyme expression in MDA-MB-468 and MCF7 cells. The data suggest that CYP1 enzymes can activate tangeretin to the more potent antiproliferative agent 4'OH tangeretin in human breast cancer cells.

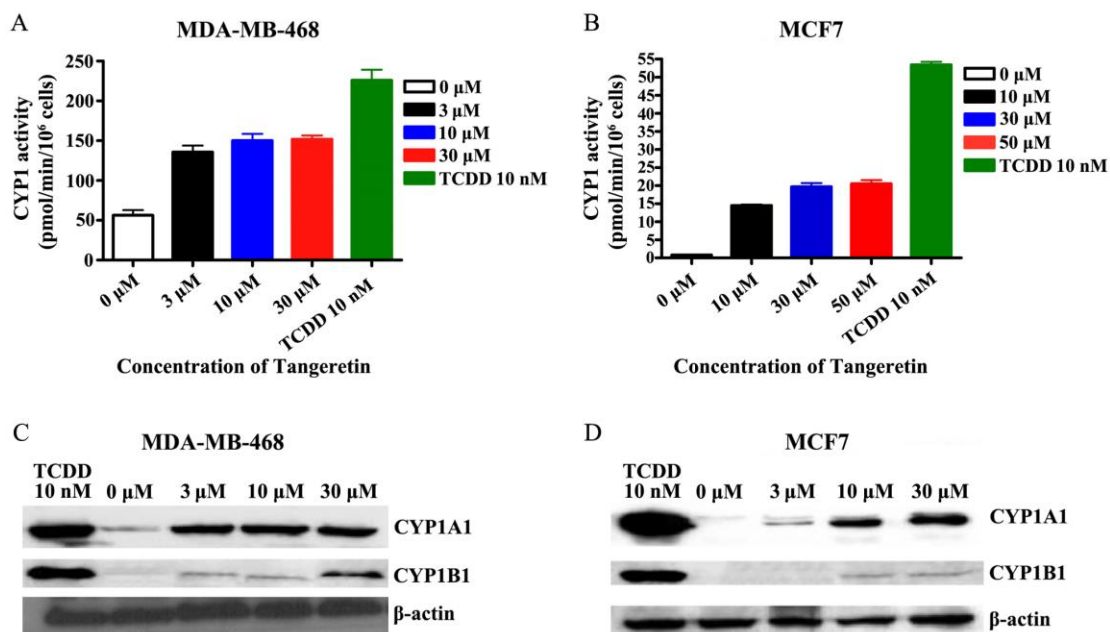


Figure 6. Tangeretin induces CYP1 activity and CYP1A1/CYP1B1 protein in human breast cancer cells. Tangeretin (3, 10, 30 μ M) was incubated with MCF7 and MDA-MB-468 cells for 24 h and CYP1 activity was measured by the EROD assay as described in materials and methods. (A) MDA-MB-468 cells (B) MCF7 cells (C) Western immunoblotting of CYP1A1/CYP1B1 protein expression in MDA-MB-468 cells pretreated with Tangeretin (3, 10, 30 μ M) for 24 h and (D) Western immunoblotting of CYP1A1/CYP1B1 protein expression in MCF7 cells pretreated with tangeretin (3, 10, 30 μ M) for 24 h. TCDD was used as a positive control at 10 nM.

The metabolism of tangeretin by CYP1 enzymes was investigated concisely in an early study conducted by Walle and Walle in 2007 (Walle and Walle, 2007). The authors examined the metabolism of a range of methoxylated flavones, including mono-, di-, tri- and fully methoxylated compounds, by human liver microsomes and recombinant CYP3A4, CYP2C9, CYP2D6 and CYP1A1/CYP1A2 enzymes (Walle and Walle, 2007). In accordance with the present study, CYP1A1 was the predominant catalyst of all compounds investigated with the exception of 5,7,4'-trimethoxyflavone that was metabolized slightly faster by CYP1A2 compared with CYP1A1. Oxidative demethylation was demonstrated as the rate-limiting metabolic reaction for fully methylated flavones, which is in agreement with the present study

and our earlier report that investigated the metabolism of the fully methylated flavone nobiletin by CYP1 enzymes (Walle and Walle 2007; Surichan et al., 2012). However, Walle and Walle did not examine the metabolism of tangeretin by CYP1B1 and did not identify the corresponding conversion products as opposed to the present study. In an earlier study conducted by Breinholt and colleagues, the identity of the CYP1A2, CYP3A4, CYP2D6 and CYP2C9–catalyzed metabolites of tangeretin was verified by LC/MS (Breinholt et al., 2003). The major metabolic routes involved one demethylation step at the 4' position of the B ring and 2 demethylation steps at the 5 and 6 position of the A ring that yielded the corresponding 4' hydroxy tangeretin and 5,6 dihydroxy tangeretin, respectively (Breinholt et al., 2003). In addition, the demethylation at the 4' position of the B ring has been previously reported as the main CYP1–mediated metabolic reaction for the structurally similar flavones nobiletin and eupatorin (Surichan et al., 2012; Androutsopoulos et al., 2008). Although the present study demonstrated that the major conversion product of CYP1A1 and CYP1B1 metabolism of tangeretin was 4' hydroxy tangeretin, the presence of additional metabolites was not evident notably in the human breast cancer cell lines MCF7 and MDA–MB–468. The possible explanations for this outcome include the reduced levels of CYP1A1/CYP1B1 enzyme activity compared with the recombinant CYP1 enzymes.

The metabolism of tangeretin to 4' hydroxy tangeretin enhanced the antiproliferative activity of the parent compound, as determined by cell viability and cell metabolism studies. Furthermore, it is important to note that the mechanism of action involved G1 arrest of MDA–MB–468 cells that was reversed in the presence of the CYP1 inhibitors acacetin and α -naphthoflavone. The data are in agreement with previous reports that demonstrated cytostatic effects of tangeretin in colon cancer, breast cancer

and yeast cells (Pan et al., 2002; Morley et al., 2007; Chong et al., 2013; Arivazhagan and Sorimuthu Pillai 2014). Induction of G1 arrest in combination with upregulation of p53/p21 and downregulation of p-Rb and cyclin D1 were caused by tangeretin treatment in rat breast cancer cells and human colon cancer cells, respectively (Arivazhagan and Sorimuthu Pillai 2014; Pan et al., 2002). Moreover, the induction of apoptosis, the inhibition of VEGF activity and the downregulation of MMP-2 and MMP-9 are additional modes of action that have been reported for tangeretin (He et al., 2015; Charoensinphon et al., 2013; Arivazhagan and Sorimuthu Pillai 2014). The present study provides additional evidence regarding the antiproliferative activity of the CYP1-metabolite 4' hydroxy tangeretin. This novel mechanism of action that includes CYP1-mediated bioactivation of tangeretin to 4' hydroxy tangeretin may apply for other types of cancer cells, such as colon cancer cells that express active CYP1 enzymes.

With regard to the potency of tangeretin in inhibiting the proliferation of human breast cancer cells, this compound exhibited high activity in MDA-MB-468 compared with MCF7 cells. By contrast, MCF10A cells were notably unaffected as the IC₅₀ of tangeretin noted in this cell line was higher than 100 μ M. Tangeretin exhibited an activation factor of approximately 72-fold in MDA-MB-468 cells compared with MDA-MB-468 cells that were treated with tangeretin and CYP1 inhibitors (18 μ M/0.25 μ M). A similar trend has been previously demonstrated for the flavonoids sinensetin, genkwanin and eupatorin in our earlier reports (Androutsopoulos et al., 2009d; Androutsopoulos et al., 2008). These compounds exhibited IC₅₀ values for MDA-MB-468 cells of 0.2, 1.6 and 0.5 μ M, respectively, whereas in MCF10A cells they were considerably less active (65, 75 and 50 μ M, respectively). In the presence of the CYP1 inhibitor acacetin, the IC₅₀ values of

sinensetin, eupatorin and genkwanin were considerably increased (from 0.2 to 13.5 μM for sinensetin, from 0.5 to 15 μM for eupatorin and from 1.6 to 12.5 μM for genkwanin) (Androutsopoulos et al., 2009d; Androutsopoulos et al., 2008). The data reported in the present study are in agreement with the aforementioned studies and suggest that the degree of methoxylation in the flavone moiety enhances the antiproliferative activity of these compounds in MDA-MB-468 cells due to cytochrome P450 CYP1-metabolism. This is verified by the observation that sinensetin and tangeretin that are both penta-methoxylated flavones exhibited submicromolar IC₅₀ values with similar activation factors in the presence of CYP1 inhibitors (68-fold vs. 72-fold).

In addition, the data indicated that tangeretin induced CYP1 enzyme activity and CYP1 enzyme expression in MCF7 and MDA-MB-468 human breast cancer cells. CYP1A and CYP2B enzyme activities and CYP1A and CYP2B enzyme expression were increased by tangeretin treatment in rat liver tissues, as demonstrated by an early study by Canivenc-Lavier et al. (Canivenc-Lavier et al. 1996). Additional studies have documented CYP1 inductive effects by the structurally similar flavones eupatorin, cirsiolol, eupatorin-5-methyl ether and nobiletin in human breast cancer cells at similar concentrations to those used for tangeretin in the current report (Androutsopoulos et al., 2009c, Androutsopoulos and Tsatsakis 2014, Surichan et al., 2012). Hydroxylated and monomethoxylated flavones such as quercetin and diosmetin, respectively, have also been shown to induce CYP1A1 mRNA and CYP1A enzyme activity via direct binding to the AhR in MCF7 breast cancer cells (Ciolino et al., 1999; Ciolino et al., 1998). It was suggested that the lipophilicity of these natural compounds that is determined by their methoxy substitutions affected their binding to the AhR and consequently the induction of CYP1 enzyme activity.

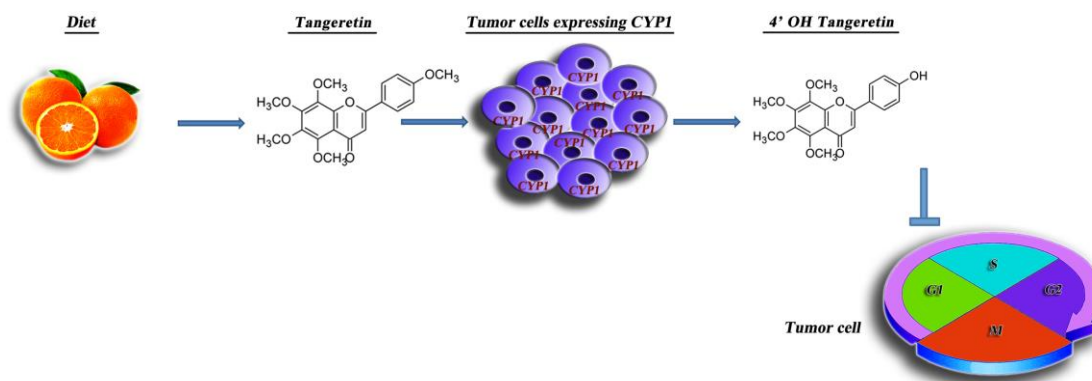


Figure 7. Putative metabolic pathway indicating the bioactivation of tangeretin in cancer cells. Tangeretin is obtained by nutritional sources and can inhibit the cell cycle arrest of cancer cells at the G1 phase by metabolism to the conversion product 4' OH tangeretin.

Our preliminary analysis suggests that tangeretin induces the expression of the AhR and its translocation to the nuclei of MCF7 cells (data not shown). It is important to note that the induction of CYP1B1 protein by tangeretin is shown for the first time as previous studies were mainly focused on the interactions of CYP1A1 and CYP1A enzymes with dietary flavones (Canivenc–Lavier et al. 1996; Ciolino et al., 1999; Ciolino et al., 1998).

In conclusion, the present study investigated the anticancer effects of tangeretin with regard to CYP1-mediated metabolism, CYP1-enzyme induction and inhibition of cellular proliferation of human breast cancer cells. The data demonstrate that tangeretin is activated in human breast cancer cells due to CYP1-mediated conversion to the metabolite 4' hydroxy tangeretin that in turn results in G1 arrest of the cells (Fig. 7). These effects were absent in normal breast cells that do not express CYP1 enzymes. The findings suggest that tangeretin could possess therapeutic implications in breast cancer cells that express active CYP1 enzymes (Fig. 7). In conclusion, tangeretin may be considered as a selective CYP1-activated natural product that can be further evaluated for its cancer therapeutic efficacy *in vivo*.

Conflict of interest

None declared.

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